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**APPLICATION NUMBER:** ***60/555,813***
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PTO/SB/16 (01-04)

# PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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| INVENTOR(S) | | |
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☐ *Additional inventors are being named on the separately numbered sheets attached hereto*

**TITLE OF THE INVENTION (500 characters max)**

**METHODS OF REFOLDING GCSF**

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*OR*

| | | | | | |
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**ENCLOSED APPLICATION PARTS *(check all that apply)***

☒ Specification *Number of Pages* 7 ☐ CD(s), Number

☒ Drawing(s) *Number of Sheets* 2 ☐ Other (specify)

☐ Application Data Sheet. See 37 CFR 1.76

**METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT**

☒ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order is enclosed to cover the filing fees

☒ The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: **20-1430**

☐ Payment by credit card. Form PTO-2038 is attached.

**FILING FEE Amount ($)** 80

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: .



*Respectfully submitted,*

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Docket Number: 019957-019700US

***USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT***

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Attorney Docket No.: 019957-019700US
Client Reference No.: NEO00261

# PROVISIONAL

# PATENT APPLICATION

# METHODS OF REFOLDING GCSF

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# METHODS OF REFOLDING GCSF

## CROSS-REFERENCES TO RELATED APPLICATIONS

**[0001]** NOT APPLICABLE

## FIELD OF THE INVENTION

**[0002]** The present invention is related to methods of refolding recombinantly produced granulocyte colony stimulating factor (GCSF), particularly GCSF produced in *E. coli* and isolated from inclusion bodies.

## BACKGROUND OF THE INVENTION

**[0003]** Many eukaryotic proteins are commercially and therapeutically useful, *e.g.*, GCSF, particularly human GCSF. The most efficient method to produce therapeutically useful eukaryotic proteins, such as GCSF, is to express the protein in bacteria. However, in bacteria, many eukaryotic proteins are expressed as insoluble proteins in bacterial inclusion bodies, and yields of active protein from the inclusion bodies can be very low. Thus, there is a need for improved methods to produce eukaryotic proteins, *e.g.*, GCSF, in bacteria. The present invention solves this and other needs.

## BRIEF SUMMARY OF THE INVENTION

**[0004]** The invention provide a method of refolding an insoluble recombinant granulocyte colony stimulating factor (GCSF) protein, by solubilizing the GCSF protein; and then contacting the soluble GCSF protein with a buffer comprising a redox couple to refold the GCSF protein, wherein the refolded GCSF protein is biologically active.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0005]** Figure 1 demonstrates production of recombinant GCSF in bacteria grown in various media and induced with IPTG.

**[0006]** Figure 2 provides Western blot analysis of refolded GCSF after SP-sepharose chromatography.

## DETAILED DESCRIPTION OF THE INVENTION

### I. Introduction

**[0007]** The present invention provides conditions for refolding GCSF proteins that are expressed as insoluble proteins in bacterial inclusion bodies. Refolding buffers comprising redox couples are used to enhance refolding of insoluble eukaryotic GCSF proteins. The refolded eukaryotic glycosyltransferases can be used as therapeutics, or alternatively can be further modified by O-linked glycosylation as described in U.S. Patent Applications 60/535284, filed January 8, 2004; 60/544411, filed February 12, 2004; and Attorney Docket Number 019957-018820US, filed February 20, 2004; each of which is herein incorporated by reference for all purposes. As used herein GCSF refers to the GCSF wild type and variant proteins described in U.S. Patent Applications 60/535284, filed January 8, 2004; 60/544411, filed February 12, 2004; and Attorney Docket Number 019957-018820US, filed February 20, 2004; each of which is herein incorporated by reference for all purposes.

### II. Refolding insoluble glycosyltransferases

**[0008]** Many recombinant proteins expressed in bacteria are expressed as insoluble aggregates in bacterial inclusion bodies. Inclusion bodies are protein deposits found in both the cytoplasmic and periplasmic space of bacteria. (*See, e.g.*, Clark, *Cur. Op. Biotech.* 12:202-207 (2001)). Recombinant GCSF proteins are expressed in bacterial inclusion bodies, and methods for refolding these proteins to produce active GCSF proteins are provided herein.

#### *A. Conditions for refolding active glycosyltransferases*

**[0009]** To produce active GCSF proteins from bacterial cells, GCSF proteins are expressed in bacterial inclusion bodies, the bacteria are harvested, disrupted and the inclusion bodies are isolated and washed. In one embodiment, three washes are performed: a first wash in a buffer at a pH between 6.0 and 9.0; a monovalent salt, *e.g.*, sodium chloride; a nonionic detergent, *e.g.*, Triton X-100; an ionic detergent, *e.g.*, sodium deoxycholate; and EDTA; a second wash in a detergent free buffer, and a third wash in H2O. The proteins within the inclusion bodies are then solubilized. Solubilization can be performed using denaturants, *e.g.*, guanidinium chloride or urea; extremes of pH; or detergents or any combination of these. In one embodiment of 5-6M guanidine HCl or urea are used to solubilize GCSF. In another embodiment, DTT is added.

[0010] After solubilization, denaturants are removed from the GCSF protein mixture. Denaturant removal can be done by a variety of methods, including dilution into a refolding buffer or buffer exchange methods. Buffer exchange methods include dialysis, diafiltration, gel filtration, and immobilization of the protein onto a solid support. (*See, e.g.*, Clark, *Cur. Op. Biotech.* 12:202-207 (2001)). Any of the above methods can be combined to remove denaturants.

[0011] Disulfide bond formation in the GCSF proteins is promoted by addition of a refolding buffer comprising a redox couple. Redox couples include reduced and oxidized glutathione (GSH/GSSG), cysteine/cystine, cysteamine/cystamine, DTT/GSSG, and DTE/GSSG. (*See, e.g.*, Clark, *Cur. Op. Biotech.* 12:202-207 (2001)). In one embodiment the redox couple is GSH/GSSG at a ratio of 10:1.

[0012] Refolding can be performed in buffers at pH's ranging from, for example, 6.0 to 10.0. Refolding buffers can include other additives to enhance refolding, *e.g.*, L-arginine (0.4-1M); PEG; low concentrations of denaturants, such as urea (1-2M) and guanidinium chloride (0.5-1.5 M); and detergents (*e.g.*, Chaps, SDS, CTAB, lauryl maltoside, Tween 80, and Triton X-100).

[0013] After refolding, the GCSF protein can be dialyzed to remove the redox couple or other unwanted buffer components. In one embodiment, dialysis is performed using a buffer including sodium acetae, glycerol, and a non-ionic detergent, *e.g.*, Tween-80. After dialysis the GCSF protein can be further purified, and/or concentrated by ion exchange chromatography. In one embodiment, an SP-sepharose cation exchange resin is used.

[0014] Those of skill will recognize that a protein has been refolded correctly when the refolded protein has detectable biological activity. For a GCSF protein, biological activity can be measured using a variety of nethods. For example, biologically active GCSF proteins are substrates for the O-linked glycosylation described in U.S. Patent Applications 60/535284, filed January 8, 2004; 60/544411, filed February 12, 2004; and Attorney Docket Number 019957-018820US, filed February 20, 2004; each of which is herein incorporated by reference for all purposes. GCSF protein activity can also be measured using cell proliferation assays or white blood cell (WBC) assays in rats. (Also described in U.S. Patent Applications 60/535284, filed January 8, 2004; 60/544411, filed February 12, 2004; and Attorney Docket Number 019957-018820US, filed February 20, 2004; each of which is

herein incorporated by reference for all purposes.) The proliferation assays and the WBC assays can be done before or after O-linked glycosylation of the refolded GCSF proteins.

**[0015]** It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a bacteriophage" includes a plurality of such bacteriophage and reference to "the host bacterium" includes reference to one or more host bacteria and equivalents thereof known to those skilled in the art, and so forth.

**[0016]** The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed. Citations are incorporated herein by reference.

EXAMPLES

## Recombinant GCSF – Expression, refolding and purification

Isolation/washing IB's:

- Harvest cells by centrifugation, discard supernatant. Results of growth on various media are shown in Figure 1.
- Resuspend cell pellet in 10mM Tris pH7.4, 75mM NaCl, 5mM EDTA –use 10ml/g (lysis buffer)
- Microfluidize cells (French press works as well)
- Centrifuge 30min, 4°C at 5,000RPM-discard supernatant
- Resuspend pellet in lysis buffer and centrifuge as above
- Wash IB's in 25mM Tris pH8, 100mM NaCl, 1%TX-100, 1% NaDOC, 5mM EDTA. Pellets are resuspended by pipetting and vortexing. Centrifuge 15min 4°C 5,000RPM. Repeat this step once more (total of two washes)
- Wash pellets two times in 25mM Tris pH8, 100mM NaCl, 5mM EDTA to remove detergents, centrifuge as above
- Resuspend pellets in $dH_2O$ to aliquot and centrifuge as above. Pellets are frozen at -20C.
- IB's are resuspended at 20mg/ml in 6M guanidineHCl, 5mM EDTA, 100mM NaCl, 100mM Tris pH8, 10mM DTT using a pipettor, followed by rotation for 2-4h at room temperature.
- Centrifuge solubilized IB's for 1min at room temperature at 14,000RPM. Save supernatant.
- Dilute supernatant 1:20 with refold buffer 50mM MES pH6, 240mM NaCl, 10mM KCl, 0.3mM lauryl maltoside, 0.055% PEG3350, 1mM GSH, 0.1M GSSG, 0.5M arginine and refold on rotator overnight at 4°C.

- Transfer refold to Pierce snakeskin 7kDa MWCO for dialysis. Dialysis buffer 20mM NaOAc pH4, 50mM NaCl, 0.005% Tween-80, 0.1mM EDTA. Dialyze a total of 3 times versus at least a 200 fold excess at 4°C.
- After dialysis pass material through a 0.45μM filter.
- Equlibrate SP-sepharose column with the dialysis buffer and apply sample. Wash column with dialysis buffer and elute with dialysis buffer containing a salt gradient up to 1M NaCl. Protein typically is eluted at 300-400mM NaCl.
- Check material on SDS-PAGE (see *e.g.*, Figure 2).

**[0017]** It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

WHAT IS CLAIMED IS:

1. A method of refolding an insoluble recombinant granulocyte colony stimulating factor (GCSF) protein, the method comprising the steps of:

(a) solubilizing the GCSF protein; and

(b) contacting the soluble GCSF protein with a buffer comprising a redox couple to refold the GCSF protein, wherein the refolded GCSF protein is biologically active.

PATENT

Attorney Docket No.: 019957-019700US
Client Reference No.: NEO00261

# METHODS OF REFOLDING GCSF


## ABSTRACT OF THE DISCLOSURE

The present invention is related to methods of refolding recombinantly produced granulocyte colony stimulating factor (GCSF), particularly GCSF produced in *E. coli* and isolated from inclusion bodies.


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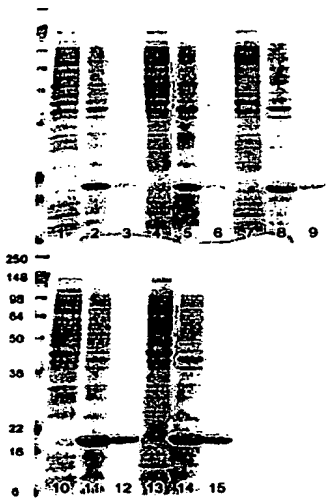
Testing growth conditions – washing IBs

| | 37°C, 1mM (final) IPTG | | | IB yields (~0.7-1g/L) |
|---|---|---|---|---|
| | Media | Buffered pH | Time | wet IB pellet (mg) |
| 1. | LB | n/a | 5hr | 120 |
| 2. | TB | 7.4 ($K\text{-}PO_4$) | 5hr | 230 |
| 3. | TB | 6 (MES) | 5hr | 170 |
| 4. | LB | n/a | o/n | 190 |
| 5. | TB | 7.4 ($K\text{-}PO_4$) | o/n | 250 |

Cell samples, loaded equally by µg
(better expression of overnights more apparent)

1. #1 lysate, supe
2. #1 lysate, pellet
3. #1 final IB
4. #2 lysate, supe
5. #2 lysate, pellet
6. #2 final IB
7. #3 lysate, supe
8. #3 lysate, pellet
9. #3 final IB
10. #4 lysate, supe
11. #4 lysate, pellet
12. #4 final IB
13. #5 lysate, supe
14. #5 lysate, pellet
15. #5 final IB




Purified IB's are shown in lanes 3, 6, 9, 12 and 15. Note IB lanes loaded at approximately ½ the amount of the preceding lane (based on predicted pellet weight).

**FIGURE 1**

## Western Blot Analysis of Refolded G-CSF

~~Native Polyacrylamide Gel Electrophoresis~~

1 2 3 4

Lane 1, Neupogen
Lane 2, G-CSF020604
(active)
Lane 3, G-CSF021004
(inactive)
Lane 4, G-CSF021804
(to be assayed)

G-CSF020604 and G-CSF021004 came from the same batch of refolding reaction. The only difference was that G-CSF021004 was saved from the flowthough of G-CSF020604 by adjusting pH and reloading onto SP Sepharose. G-CSF021804 was a separate refolding batch.

After SP-sepharose you see only one band by Coomassie stain or Western.

# FIGURE 2